Evaluation of the Efficacy of DNA Sequencing and Microhistological Analysis for Determining Diet Composition in Ungulates

by

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ABSTRACT

An understanding of diet habits is crucial in implementing proper management strategies for wildlife. Diet analysis, however, remains a challenge for ruminant species. Microhistological analysis, the method most often employed in herbivore diet studies, is tedious and time consuming. In addition, it requires considerable training and an extensive reference plant collection. The development of DNA barcoding (species identification using a standardized DNA sequence) and the availability of recent DNA sequencing techniques offer new possibilities in diet analysis for ungulates. Using fecal material collected from controlled feeding trials on pygmy goats, (*Capra hicus)*, novel DNA barcoding technology using the P6-loop of the chloroplast *trn*L (UAA) intron was compared with the traditional microhistological technique. At its current stage of technological development, this study demonstrated that DNA barcoding did not enhance the ability to detect plant species in herbivore diets. A higher mean species composition was reported with microhistological analysis (79%) as compared to DNA barcoding (50%). Microhistological analysis consistently reported a higher species presence by forage class. For affect positive species identification, microhistology estimated an average of 89% correct detection in control diets, while DNA barcoding estimated 50% correct detection of species. It was hypothesized that a number of factors, including variation in chloroplast content in feed species and the effect of rumen bacteria on degradation of DNA,

influenced the ability to detect plant species in herbivore diets and concluded that while DNA barcoding opens up new possibilities in the study of plant-herbivore interactions, further studies are needed to standardize techniques and for DNA bar-coding in this context.

DEDICATION

This thesis is dedicated to my family, near and far: To my father who shared his deep, abiding respect for science and nature; my mother who encouraged me to pursue my goals and fight for what I believe; my siblings, who offered love and support; but especially my children, Kyle, McKenzie, Delaney and Cayden, a constant source of joy and pride; and my loving husband, Patrick, who will always be my rock.

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CHAPTER 1

INTRODUCTION

A thorough understanding of ecosystem functioning is essential in the application of techniques necessary for proper wildlife habitat management (Duffy et al. 2007). Where endangered species are concerned, the study of feeding ecology becomes crucial as a precise knowledge of the animal's diet must be gathered in order to design a reliable conservation strategy (Marrero et al.2004, Cristóbal-Azkarate and Rodrígez 2007, Valentini et al. 2009). Assessment of feeding habits within an ecosystem relies on the basic underlying principle of wildlife habitat evaluation and assumes the following: 1) When adequate cover, water and space are provided, the physical well-being of the wildlife species under consideration is a function of the quality and quantity of the diet; and 2) An individual maintained on a high nutritional plane is more productive and less subject to losses due to natural causes (Nelson and Leege 1982).

In order to fully understand the factors affecting species requirements it is necessary to determine: 1) components of the habitat utilized by the individual species, 2) quantity of each component utilized by the individual animal under consideration, and 3) the nutritional quality of the diet. Large herbivore species will select specific diets among a wide availability of food plants due to differences in digestion capacity, tolerance to fibers or secondary compounds, and energy requirements (Shipley 1999). Such complexity in the nature of the

determinants of food choices by herbivores, as well as the response of plant species or plant communities to herbivory leads to great difficulty in characterizing the plant-herbivore relationship (Giles et al. 2011, Howe 1988, McInnis et al. 1983).

In addition to the ecological applications for diet analysis in ungulates, heightened public awareness regarding the origin of food products has increased the demand for strict specifications on animal breeding due to recent food scares (e.g. bovine spongiform encephalopathy and avian flu) and the use of genetically modified organisms for feeding of livestock (Pegard et al. 2009, Pascal and Mahe 2001, Pinotti et al. 2005). Certifying husbandry conditions with guaranteed quality labels assumes that analytical tools exist with the ability to authenticate the processes used, especially the animal diet (Pegard et al. 2009.)

Microhistological analysis, the method most often employed in herbivore diet studies, is tedious and time consuming. In addition, it requires considerable training and an extensive reference plant collection (Holechek and Gross 1982a). Furthermore, absence of a unique combination of epidermal features in some species prohibits irrefutable identification (Henley et al. 2001, Smith et al. 2002). Novel DNA-based analysis of fecal samples may prove a useful and fast tool for estimation of herbivore diet (Taberlet et al. 2007,Valentini et al. 2009). This technique allows for precise identification of amplified DNA sequences, usually to the species level (Valentini et al. 2008).

The objectives of this project are 1) to determine whether DNA barcoding can enhance the ability to detect plant species in herbivore diets and 2) to evaluate the efficacy of DNA barcoding technology and microhistology for the determination of diet composition of a ruminant animal.

CHAPTER 2

LITERATURE REVIEW

Methods of Diet Analysis

Ideally, a method for determining grazing-animal diets will allow free animal movement and complete natural selection of all available plants and plant parts regardless of pasture size, allow for diet determination regardless of terrain, be equally useful for wild and domesticated animals, not require slaughter of test animals, require a minimum of animal care, be relatively objective, and allow identification of each individual plant species consumed (Sander et al. 1980).

Numerous analysis techniques have been employed over the past 60 years to quantitatively or qualitatively evaluate the diet composition of large ungulates including: Direct evaluation of plants eaten by the animal from observation in the field (Hubbard 1952, Bjugstad et al.1970), identification of herbivory impacts directly on plant species, (Edlefsen et al. 1960, Smith et al. 1962) near infrared reflectance spectrometry (Foley et al.1998, Kanek and Lawler 2006), quantification of n-alkanes (Newman et al. 1998, Ferreira et al. 2007, Piasentier et al. 2007), stable isotope analysis (Codron and Brink 2007, Sponheimer et al. 2003), fistula sampling (Vavra et al. 1978, Rice 1970), stomach and intestinal tract analysis (Bertolino et al. 2009, Norbury and Sanson, 1992), microhistological analysis (Morrison et al. 2009, Eckerle et al.2009, Johnson et al.

2009, Shrestha and Wegge 2006, Tafoya et al. 2001, Holechek et al. 1982), and most recently DNA barcoding (Giles et al. 2001, Ho et al. 2009, Kowalczyk et al. 2011, Pegard et al. 2009, Poinar et al. 1998, Soininen et al. 2009).

Associated with each of these methods are a number of advantages and disadvantages which have stimulated discussion as to which is most useful in interpreting food habits of large herbivores (Giles et al. 2011, Valentini, 2009, Alipayo et al. 1992).

Microhistological Analysis

The application of fecal pellets for microhistological analysis is most commonly used in wildlife and range studies to determine diet composition by identifying plant tissues and recording frequency counts of fragments in collected samples (Aiken 1989, Johnson et al. 1983). Holechek and Gross (1982a) provide a comprehensive review of this procedure.

Microhistological analysis has several unique advantages that account for its popularity as a research tool (Holechek and Gross, 1982b, Scotcher, 1979). These advantages include the fact that fecal analysis: (1) Does not interfere with the normal habits of the animals; (2) permits practically unlimited sampling; (3) places no restriction on animal movement; (4) has particular value where animals range over mixed communities; (5) is the only feasible procedure to use when

studying secretive and/or endangered species; (6) can be used to compare diets of two or more animals at the same time; and (7) requires very little equipment.

Disadvantages of this technique have also been reviewed (Holechek et al. 1982, Vavra and Holechek 1980) and include the following: (1) considerable labor and time for actual analysis because an extensive reference plant collection is required and the observer must have extensive training in order to accurately identify plant fragments; (2) differences in fragmentation of plant species during slide preparation; (3) absence of a unique combination of epidermal features in some species (especially dicots) which prohibits unequivocal identification at the species and sometimes at the genus level (Henley et al, 2001, Smith et al.2002); (4) differences in digestibility of species which lead to an underestimation of the quantity of forbs consumed and over estimation of grasses; and (5) difficulties in estimating diets of herbivores consuming various quantities of woody materials throughout the year as woody plant parts have lower proportions of identifiable epidermal material than leaves or young stems (Westoby et al. 1997, Holechek and Valdez 1985, Alipayo et al. 1992).

However despite its limitations, fecal analysis (with a number of modifications) has been considered the most reliable method for evaluating wild herbivore diets in many situations and continues to be widely used today (Morrison et al. 2009, Shrestha and Wegge 2006, Tafoya et al. 2001). According to Vavra et al. (1978) and Aiken (1989) this technique has provided an accurate

and precise method to qualify herbaceous plant intake and species composition. More recent studies have confirmed that with proper sampling and adequate technician training, actual diet composition and estimated diet composition using microhistological analysis can approach a ratio of 1:1 (Paola et al. 2005).

DNA Sequencing

The development of rapid , accurate and automatable species identification using a standardized DNA sequence (DNA barcoding) and the availability of recent DNA sequencing technology offer new possibilities in diet analysis (Hebert and Gregory 2005, Valentini et al. 2009). Numerous DNA-based studies have been successful in determining diet composition, using amplifiable DNA extracted from feces, either for carnivores using mitochondrial DNA (Hofreiter e. al. 2000, Passmore et al. 2006, Bradley et al. 2007, Deagle et al. 2005, Deagle et al. 2007, Clare et al. 2009) or for herbivores using chloroplast DNA (Giles et al. 2001, Ho et al. 2009, Kowalczyk et al. 2011, Pegard et al, 2009, Poinar et al. 1998, Soininen et al. 2009).

According to Valentini and Taberlet (2008) DNA barcoding is advantageous when food habits are not identifiable by morphological criteria, such as in liquid feeders, (Agusti et al. 2003) and can also provide valuable information when the diet cannot be deduced by observing the eating behavior, such as in the case of or giant squid *(Architeuthis spp.)* in the sea abyss (Deagle et al. 2005). Current techniques allow species identification based on the

amplification and analysis of DNA even from degraded organic substrates (Teletchea et al. 2005) and has also been applied in forensics (Capelli et al. 2003; Wan et al. 2003) the analysis of fossils (Hofreiter et al. 2000,) ecology (Ficetola et al. 2008) and the food industry (Maudet and Taberlet 2002).

For purposes of species identification in diet analysis, DNA barcoding entails amplifying suitable markers (gene regions) from dietary samples using polymerase chain reaction (PCR), sequencing the resultant amplicons and identifying the sequences by comparison to a reference database (Deagle et al. 2010). Species identification can be set up using sets of specific primer pairs, each pair amplifying DNA (if present) from a single species or group of closely related species. The presence/absence of DNA from each species or group is then detected by the success of the corresponding PCR amplification (Deagle et al. 2007). Universal primer pairs have also been used, allowing the amplification of a given DNA fragment for a large set of species in a single PCR reaction (Bradley et al. 2007). Species identification is then possible by analyzing the variability of the fragments amplified.

The selection of the ideal DNA barcoding marker is crucial and should meet several criteria (Valentini et al. 2009). First, it should be sufficiently variable to discriminate among all species, but conserved enough to be less variable within, than between, species. Second, it should be standardized, with the same DNA region as far as possible used for different taxonomic groups.

Third, the target DNA region should contain enough phylogenic information to easily assign species to a taxonomic group (genus, family, etc.). Fourth, it should be extremely robust, with highly conserved priming sites, and highly reliable DNA amplification and sequencing. Fifth, the target DNA region should be short enough to allow amplification of degraded DNA (Taberlet et al. 2006, Valentinni et al. 2009). According to Valentini et al. (2009) this "ideal barcode" does not yet exist.

The now well-established Consortium for the Barcode of Life (CBOL), an international initiative supporting the development of DNA barcoding, aims to promote global standards and coordinate research in DNA barcoding (Valentini and Taberlet 2008). For animals, the gene region proposed for the standard barcode is a 658 base pair region in the gene encoding the mitochondrial cytochrome C oxidase 1 (CO1) (Hebert et al. 2003). 16S *rRNA*, another mitochondrial gene in addition to CO1, or nuclear ribosomal DNA (nrDNA) has also been proposed as useful barcoding markers (Hollingsworth et al. 2011). As yet, there is no consensus in the scientific community for additional markers. In plants the situation is more controversial due, in part, to the fact that both the mitochondrial and chloroplast genomes are evolving too slowly to provide enough variation (Hollingsworth et al. 2009). Many strategies have been proposed for plants, either based on a single (group-specific) chloroplast region (Kress et al. 2005, Lahaye et al. 2008) or on a combination of different regions utilizing

universal primers (Bradley et al. 2007, Chase et al. 2007). Recently, it has been proposed to use three coding chloroplast DNA regions that together would represent the standard barcode: *rpo*C1, *mat*K, and either *rpo*B or *psb*A-*trn*H (Chase et al. 2007). Ordinarily, the use of specific primers requires advance knowledge of the animal's diet. This is not possible in most cases and makes the universal approach more appropriate (Valentini et al. 2008). Deagle et al. (2007) utilized both group-specific and universal primers when analyzing the diet of Macaroni penguin *(Eudyptes chrysolophus)* using feces as a source of DNA. The results obtained with five different sets of specific primers were similar to those involving a universal mitochondrial gene (16S rDNA), supporting the relevance in the use of universal primers for diet analysis. Hollingsworth et al. (2009) provide an historical overview of the continuing search for an appropriate plant barcode.

Potential identification problems utilizing DNA barcoding have been extensively discussed (Rubinoff 2006). One of the major limitations for the barcoding approach is the fact that identical mitochondrial or chloroplast DNA sequences can be present in different related species due to introgression, or due to incomplete lineage sorting since the time of speciation (Ballard and Whitlock 2004). Furthermore, nuclear copies of fragments of mitochondrial or chloroplast DNA are common and can be preferentially amplified in some circumstances (Zhang and Hewitt 1996) leading to potential identification errors. Finally,

heteroplasmy (genetically different mitochondria or plastids within one cell) can also confuse the identification system (Kmiec et al. 2006). In addition, if the reference database is not comprehensive, that is, it does not contain all the species of the group under study a large percentage of misidentification may be observed (Meyer 2005).

Another shortcoming of the current status of DNA barcoding technology lies in the length of the sequences used, usually >500 base pairs (Hebert et al. 2003), which prevents the amplification of degraded DNA. Unfortunately, much potential DNA barcoding application can only be based on degraded DNA. It is usually difficult to amplify DNA fragments longer than 150bp from such samples (Deagle et al. 2006). As a consequence, there is a need for shorter barcoding markers (Taberlet et al. 2007, Hajibabaei et al. 2006, Meusnier et al. 2008).

Recently, Taberlet et al. (2007) designed a pair of primers targeting the P6 loop of the chloroplast *trn*L (UAA) intron. This fragment is adequate for the identification of DNA remaining in feces because the primers are universal in plants (i.e., highly conserved for angiosperms and gymnosperms), and the short size of the target fragment (10-143 base pairs without priming sites) allows the study of degraded DNA (Pegard et al. 2009). The targeted sequences are more conserved than those previously used for a short *rbc*L fragment (Poinar et al. 1998) and are thus more pertinent for diet analysis. Furthermore, the identification of plants is efficient because the amplified region is one of the most variable

systems in size and sequences known to date (Pegard et al. 2009). Valentini et al. (2009) showed that the *trn*L approach combined with large-scale pyrosequencing is efficient for analyzing the diet of various herbivore species, including mammals, birds, insects, and mollusks, using feces as a source of DNA. Soininen et al. (2009), demonstrated that the use of this DNA-based approach was a huge step forward in terms of taxonomic resolution, opening possibilities to answer questions about plant-herbivore interactions and diet selection more precisely than using traditional methods. According to Valentini et al. (2009) about 50% of the taxa can be identified to species using the *trn*L approach. Their research indicates that this method is quick, easy to implement and can be applied across a wide range of herbivorous species (Valentini et al. 2009).

CHAPTER 3

METHODS

Experimental Design

In order to address the objectives of this study, a series of feeding trials was performed using four diets of known composition, fed to eight pigmy goats (*capra hicus)* using a randomized block (Latin square) design. The fecal material was collected after each goat was randomly exposed to a diet mixture for 14 days. The fecal material was analyzed to determine plant species composition using both DNA barcoding and microhistological techniques, and these data were then analyzed to determine the efficacy of each technique to describe the appropriate diet composition.

Diet Formation

Twenty major forage plants were collected near Cordes Junction, AZ (Perry Mesa), in Queen Creek and Tempe, AZ from August 2010 to May 2011. Only above ground portions were collected. Species identification was confirmed by Dr. William Miller (ASU), Dr. Kelly Steele (ASU), and Dr. Andrew Salywon (Desert Botanical Garden, Tempe, AZ, Appendix A, Table 1). Names used throughout this thesis follow those used in the USDA PLANTS database (http://plants.USDA.gov).

Voucher specimens were obtained and collected forage species were stored in burlap bags and allowed to air dry. All forage species were chopped to a particle size of 3 cm and weighed. Four diet mixtures were created to reflect known proportion of grasses, forbs, and shrubs (Appendix A, Table 2). To ensure adequate nutrition, 60 - 70% of each diet consisted of alfalfa hay (*Medicago sativa*) that was prescreened for composition and purity.

Feeding Trial

The feeding trials were performed using a total of eight pygmy goats (four female and four male with a mean weight of 33.7 kg). Goats were divided into four groups of two goats each based on weight. Goats were provided by a local Gilbert, AZ farmer and housed and maintained in separate pens by the owner. Pens were constructed from hog panels; each pen measuring 1 m in height, 2.5 m in width, and 5 m in length. The day prior to the initiation of the feeding trial, pens and surrounding areas were cleared of any signs of plant material (trees, weeds, grasses etc.), then hosed down for dust control. Goats were grouped based on dominant/submissive relationships and weight (nannies were housed next to kids and dominant males were never housed next to each other) and were placed into one of four groups and randomly assigned to an initial diet . The premixed diets were offered in an individual feeder at a rate of 30 g/kg of body weight. Goats were fed twice daily and water was provided *ad libitum* for 14 days. On the evening of day 13, pens were cleaned of all fecal material. On the morning of the

fourteenth day, animals were fed final allotment of the current diet and observed in order to collect fresh fecal pellets from individual goats. Thirty grams of fresh fecal material per goat was collected and frozen for later use in microhistological and DNA processing. Goat groups were then assigned to a new diet and the trial repeated until all goats had been assigned to all four diets.

Microhistological Analysis

All species contained in the diet were processed to voucher slides in accordance with Davitt and Nelson (1982) and Holt and Miller (1992). Individual plant species were thoroughly investigated at 100X and 400X for different diagnostic features and an identification key was created. Diagnostic feature descriptions were obtained from Metcalfe (1960), Johnson et al. (1983), Green et al. (1985) and Mauseth (1988). Fecal material from each goat by feeding trial was preserved on microscope slides according to methods outlined by Davitt and Nelson (1980). Six slides were made for each diet. A 10 x 10 grid mounted on the ocular of the microscope to facilitate determination of the percent cover of each identifiable plant species and cell fragments at 100x magnification. The relative cover of each identifiable species was determined for 50 random fields on each slide, for a total of 300 fields per diet. Percent species composition was determined by taking the total cover values for each species and dividing them by the summed cover values for all species. Cover values normalized for microscope

slide density according to Drake (2009) by dividing the total cover of the replicate with largest total cover by the total cover of replicate being adjusted.

To compensate for differential digestibility of different plant species, all forage plants within each diet were subjected to an *in vitro* digestion according to Tilly and Terry (1963) as modified by Goering and VanSoest (1970). A correction coefficient for digestibility of each available forage species was developed using calculations from Drake (2009), and an average for each forage class was applied to species that were found in the diets.

DNA Sequencing

Extraction, amplification and sequencing of the P6 loop of the chloroplast *trn*L (UAA) intron, from both the fecal pellets and from the reference specimens, was performed by research scientists at the Desert Botanical Gardens (Phoenix, AZ). A Desert Botanic Garden reference plant library was established by sequencing DNA from collected plant specimens. Immediate freezing allowed storage of field specimens for up to two weeks prior to processing. Total DNA was extracted utilizing DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Plant reference samples were processed utilizing Sanger sequencing (ASU, Tempe, AZ). The Desert Botanical Plant reference library and Genbank (http://www.ncbi.nlm.nih.gov/nuccore/) were

utilized as a reference barcodes for diet analysis. Next generation sequencing of the *trnL* P6-loop fragments that were amplified from fecal pellets was performed on the Ion Torrent platform at Arizona State University DNA Laboratory.

DNA sequences were reported as frequency data (number of DNA sequences per species) and converted to percent by dividing the individual number of DNA sequences per species by the total number of DNA sequences per diet. Percent values were then multiplied by normalized cover value obtained with fecal data (Drake 2009) utilizing the following equation:

$$DNA_i \text{ Cover} = (DNA_i / \sum DNA_i) * 1140$$

where DNA_i is the number of DNA fragments for species *i*, $\sum DNA_i$ is the sum of all fragments found in the individual fecal sample, and 1140 represents the fecal diet replicate with largest total cover by the total cover of replicates being adjusted.

Statistical Analysis

The objective of this study was broken down into three levels in order to answer the overall objective of evaluation of the efficacy of DNA sequencing and microhistological analysis to determine the diet of a ruminant animal. The first was to determine if the two techniques could accurately identify the total number of species present in each of the control diets. To answer this question, a randomized block, two factor factorial design (with diet and method as factors)

was used in analysis of variance (ANOVA) (Zar, 1999). The hypothesis tested was that there was no difference in the total number of species found in the control diet, DNA barcoding, or microhistological analysis. A second part of this question was whether there was any difference in species presence detection by forage class. The hypothesis tested was that there was no difference in the total number of species by forage class found in the control diet, DNA barcoding, or microhistological analysis. This was examined using a randomized block three factor factorial (with diet method and forage class as factors) ANOVA (Zar, 1999). Significant means were separated using Tukey's mean separation test at $P \leq 0.05$

The third portion of the question was to determine if the two techniques could accurately identify the proportion of each species present in the control diets. The experimental design in this portion of the study was a randomized block three factor factorial with diet, method, and plant species as factors. The statistical hypothesis for this question was that there were no differences in proportion of each plant species found in the fecal material by method for each of the four possible diets by plant species combinations. Because the data in this portion of the study did not fit a normal distribution and could not be transferred to do so, a Friedman's rank sum test for nonparametric analysis of variance (ANOVA) was used to evaluate the data. Significant difference between means was determined using a Tukey's mean separation test (Zar 2008). All statistical

analyses were performed using “R®” statistical software. All references to statistical significance imply differences at $P \leq 0.05$.

CHAPTER 4

RESULTS

Species Presence Determination

The first portion of our research question was to determine if microhistology and DNA barcoding could accurately identify the total number of species present in each of the control diets. Results for the accuracy of the determination of plant species presence in the diet are presented in Appendix B, Table 6. Significant differences were found in detection of plant species presence between methods and between methods by diet. As seen in Table 1, microhistological analysis had significantly higher mean species presence than DNA barcoding across all four diets, and correctly detected an average of 12.25 (76%) of the original 16 species present in the control diets. DNA barcoding correctly identified an average of 8 (50%) of the original 16 species present in each of the 4 control diets.

Significant differences were also reported for control diets. This was expected as individual diets were created to reflect such differences. Percent grasses in control diets varied from 8-14%, Forbs varied from 9-16% and shrubs varied from 8-9% (Appendix B, Table 6).

Table 1. Mean species richness by method and diet used to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Diet	Control	Microhistology	DNA
Diet 1	16[a]	12.5[c]	8[e]
Diet 2	16[a]	14.25[b]	8[e]
Diet 3	16[a]	12.5[c]	8[e]
Diet 4	16[a]	9.75[d]	8[e]
Method Mean[†]	16[a]	12.25[b]	8[c]

[a..e] Means with different letters are significantly different at $P \leq 0.05$
Tukey Mean Separation Value 1.1885.

Forage Class Detection

The second portion of our research question was to determine whether there was any difference in species presence detection by forage class. A total of 16 species, consisting of five grasses, six forbs and five shrubs, were utilized in each of the control diets (Appendix A, Table 2). Results of the statistical analysis of species detection by forage classes by microhistology and DNA barcoding methods are presented in Appendix B, Table 7. Significant differences were found between methods in detection of number of species within forage classes and between method by diet. As shown in Figure 1, microhistology correctly detected an average of 4.8 of five species within the grass category (96%), 3.8 of six forbs(64%) and 4.1 of five shrubs (82%). DNA barcoding correctly detected 2.4 of 5 species within the grass category (48%), 2.5 of 6 within the forb category (42%) and 2.3 of 5 species within the shrub category(46%).

In addition to correctly identified species, false positive identification of species (species not included within control diets) were noted for both methods (Table 2). Microhistology falsely detected an average 0.19 (3.8%) of species within grass category and 0.38 (6.3%) of species within the forb category, with no false positive detection within the shrub category. DNA barcoding falsely detected an average of 0.25 (5%) of species within the grass category, 2.25 (37.5%) in forbs and 0.25 (5%) in shrubs (Table 2.).

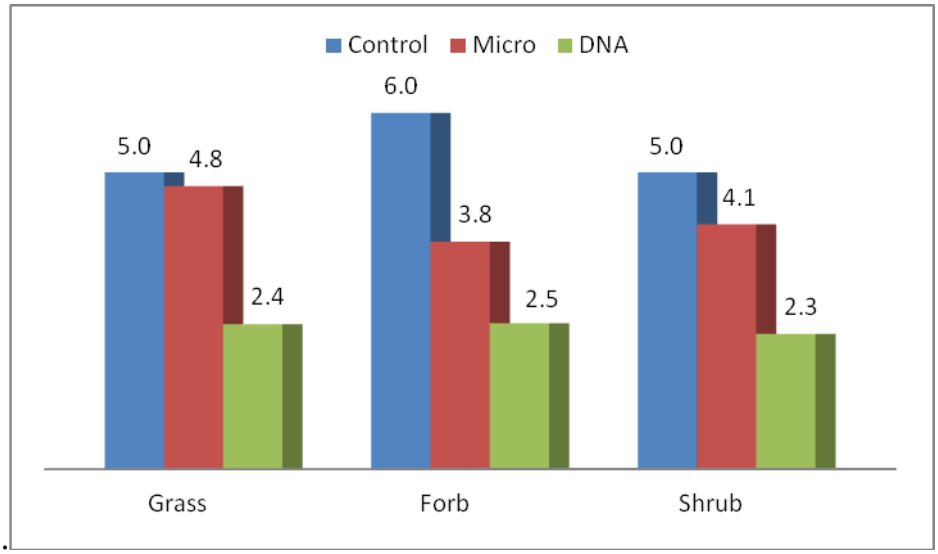

Figure 1: Mean species identification of forage class by method and diet used to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Table 2: Number of false positive identification of species by forage class used to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Method	Forage Class	Number of Species in Control	Mean False Positives
Microhistology	Grass	5	0.19
	Forb	6	0.38
	Shrub	5	0.00
DNA Barcoding	Grass	5	0.25
	Forb	6	2.25
	Shrub	5	0.25

Species Composition by Forage Class

The third portion of our research question was to determine if the two techniques could accurately identify the proportion of each species present in the control diets. Because the data in this portion of the study did not fit a normal distribution, a Friedman's rank sum test for nonparametric analysis of variance (ANOVA) was used to evaluate the data. The only significant differences occurred between DNA barcoding and control diets (Appendix B, Table 6). There was no difference in overall species quantification between microhistology and control diets. Ranked means of species composition of known diets were 54.13 for Control, 54.29 for Microhistology and 38.35 for DNA barcoding. (Appendix B, Table 10 and 11).

Figure 2 illustrates the mean % species by forage class of all goat diets detected by fecal analysis and DNA barcoding. Grasses (averaging 11% of control diets) were overestimated by microhistology (detected at 23%) and

underestimated with DNA barcoding (detected at 6%). Forbs (averaging 11% of control diets, excluding Alfalfa) and shrubs(8% of control diets) were slightly underestimated with Microhistology at (6% and 5%) and overestimated with DNA barcoding (26% for each forage class).

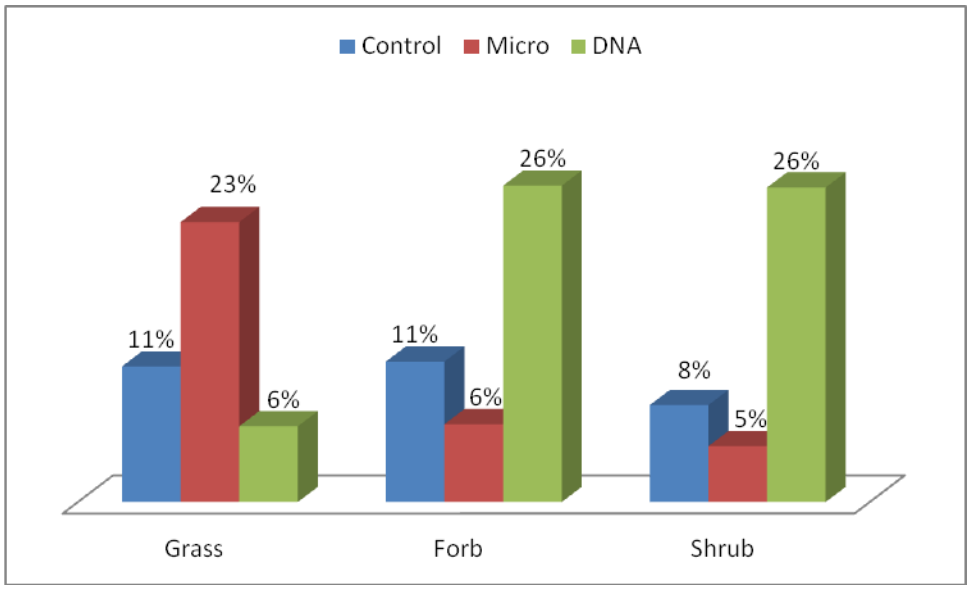

Figure 2: Mean percent species by forage class of all goat diets detected by fecal analysis and DNA barcoding.

Figures 3, 4, 5 and 6 illustrate that microhistology closely reflects the changes in species composition present in control diets with a few rare exceptions. *Bouteloua gracillis* in diets 1-3 was overestimated and *Hordeum jubatum* was overestimated in diet 4. DNA barcoding also demonstrated significant overestimation in several species including *Eschscholzia californica, Helianthus annuus, Sisymbrium irio,*and *Sonchus oleracea.*

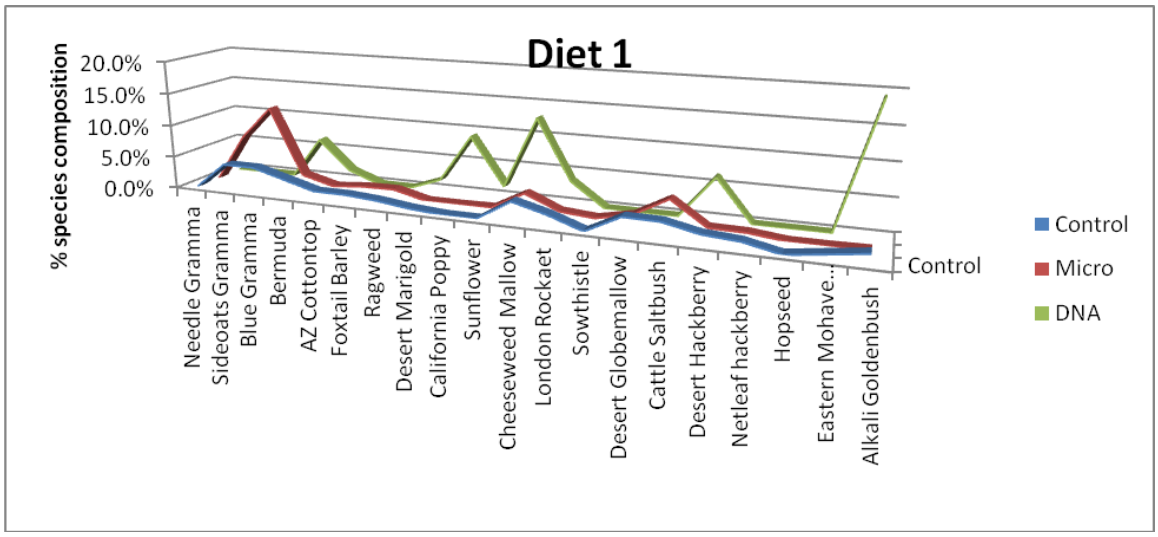

Figure 3. Diet 1 mean percent species composition of goat diets detected by fecal analysis and DNA barcoding

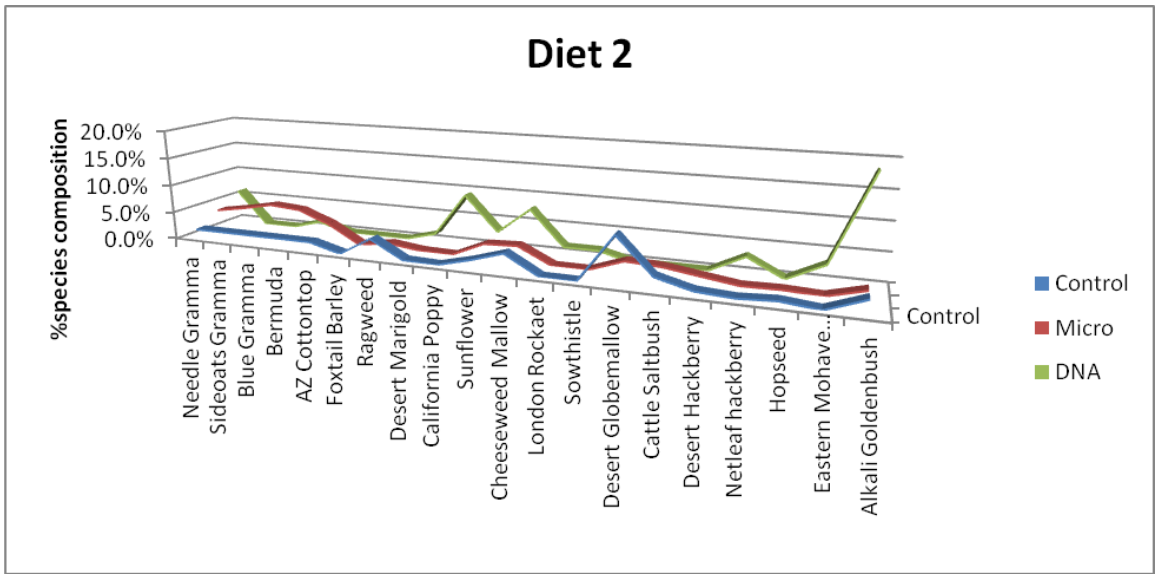

Figure 4. Diet 2 mean percent species composition of goat diets detected by fecal analysis and DNA barcoding

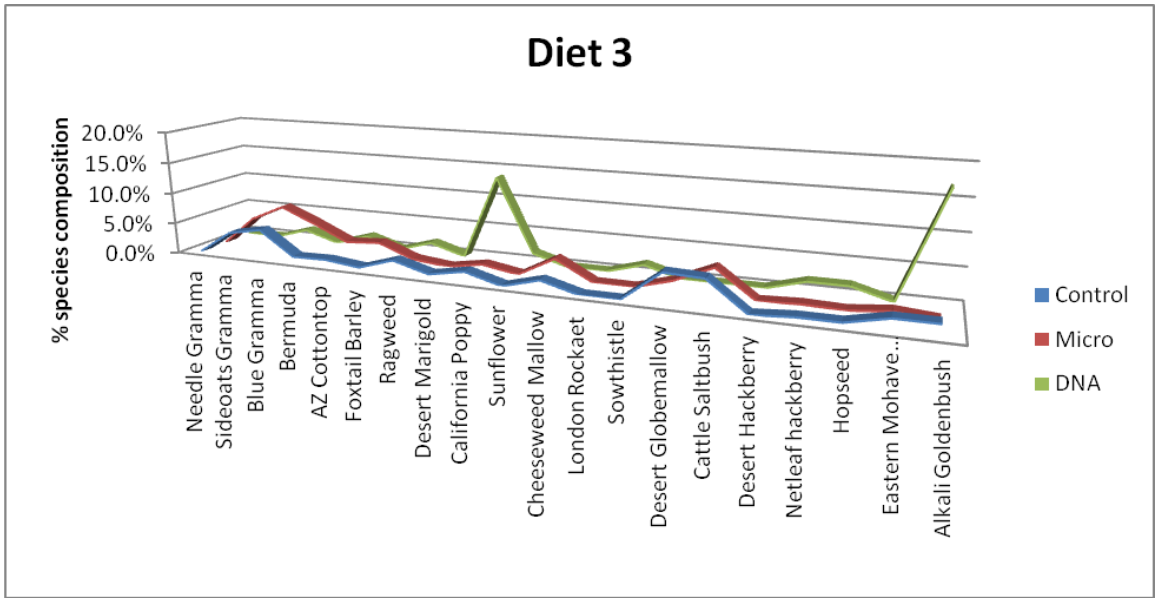

Figure 5. Diet 3 mean percent compostition of goat diets detected by fecal analysis and DNA barcoding

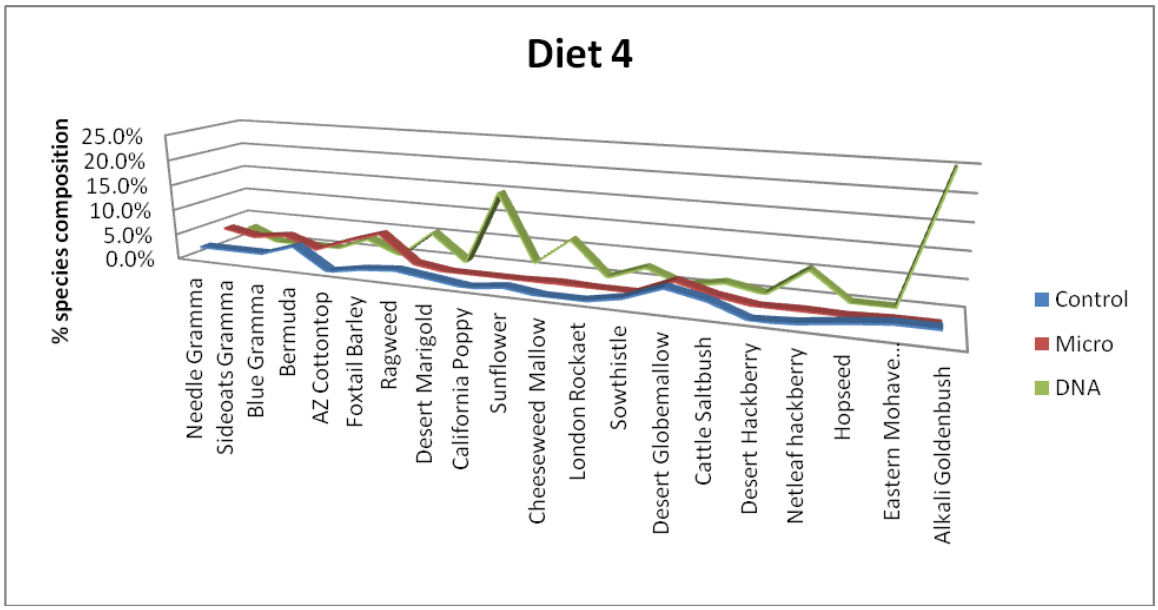

Figure 6. Diet 4 mean percent compostition of goat diets detected by fecal analysis and DNA barcoding

CHAPTER 5

DISCUSSION

DNA barcoding has attracted attention with promises to aid in species identification and discovery; however, few well-sampled datasets are available to test its performance (Meyer et al. 2005). Previous studies have analyzed herbivore diets utilizing DNA barcodes, (e.g. Taberlet et al. 2007, Valentini et al. 2009, Soininen et al. 2009). But, to our knowledge, this study represents the first results from a controlled feeding trial for ruminant species. The goals of this project were 1) to determine whether DNA barcoding could enhance the ability to detect plant species in herbivore diets and 2) to evaluate the efficacy of DNA sequencing and microhistological analysis for the determination of plant species presence and diet composition of a ruminant animal using fecal material.

At its current stage of technological development this study's findings demonstrated that DNA barcoding did not enhance the ability to detect plant species in herbivore diets. For positive species identification, microhistology estimated an average of 89% correct detection in control diets, while DNA barcoding estimated 50% correct detection of species. With regard to forage class identification, microhistology correctly detected an average of 96% grasses, 64% forbs and 82% shrubs while DNA barcoding correctly detected 48% within the grass category, 42% within forb category and 46% within shrubs.

Differences in detection of species identified by DNA barcoding for diet analysis are undoubtedly affected by the animal and type of diet consumed. The results presented in this research are inconsistent with previous studies (Soininen et al. 2009; Valentini et al. 2008) which reported that DNA based diet analysis is taxonomically more precise than microhistological identification of plant cuticles for herbivores. Soininen et al. (2009) employed DNA barcoding using the same molecular maker used in this study, P6 loop of the chloroplast trnL -UAA- intron, and microhistology to analyze stomach contents of two ecologically important subarctic herbivores species, *Microtus oeconomus* (tundra vole)and *Myodes rufocanu* (grey red-backed vole). Although the identified taxa in the diets matched relatively well between the two methods, DNA barcoding gave by far a taxonomically more detailed picture. Using DNA barcoding, 75% of all sequences were identified at least to the genus level, whereas with the microhistological method, less than 20% of the identified fragments could be specified at that level.

The vast majority of applications of DNA barcoding to diet analysis have been conducted on carnivorous species (e.g. Fin whale, (*Balaenoptera physalus)* (Jarmen et. Al 2004); insects (Pons 2006, Symondson 2002); red bat (*Lasiurus borealis)* (Clare et al. 2009); penguin (*Eudyptula minor)* and sea lion (*Eumetopias jubatus*) (Deagle et al. 2005, 2009). Other studies on diet analysis conducted with DNA barcoding involved monogastric (non-ruminant)

herbivores (e.g. voles (*Microtus oeconomus*) (Soininen et al. 2009); bear *(Ursus arctos*) and marmot (*Marmot caudata*) (Valentini et al. 2008); kangaroo (*Micropus rufus*) (Ho et al. 2009), and white colobus monkeys (*Colobus quereza*) (Bradley et al. 2007). Very few studies utilizing DNA barcoding have involved ruminant species (e.g. grazing livestock, (Pegard et al. 2009), Bison (*Bison bonasus*) (Kowalczy et al. 20120); and chamois (*Rupicapra rupicapra*) (Raye et al. 2010). Selected DNA barcoding diet studies for carnivores, non-ruminant herbivores and herbivores are highlighted in Table 3 and illustrate the mean % of DNA sequences identified to species level within each type of organism. The greatest success rate of species identification in diet studies utilizing DNA barcoding (89%) is found within studies conducted on carnivores, followed by non-ruminant herbivores (56.6%) and ruminants (29%). The successful extraction of DNA (sequences long enough for species identification) from fecal samples of each of these diverse types of feeders could vary greatly due to differences in the degree of DNA degradation during the digestion process within each category of feeder. This might possibly lead to a discrepancy in findings between ruminant and non-ruminant studies and the ability of DNA barcoding to estimate percent species composition of diets in ruminants. The exact nature of the rumen bacteria and its effect on degradation of DNA has yet to be determined.

The potential presence of DNA in a ruminant's fecal material depends largely on its survival as it passes through the digestive tract and is exposed to

nucleases (Dale et al. 2002). Monogastic (simple stomached) animals are unable to digest cellulose, but ruminant animals provide a habitat, the rumen, for anaerobic organisms that have the ability to do so (Huntgate 1966). Digestion in ruminants involves a series of processes in the alimentary tract by which feeds are broken down in particle size and finally rendered soluble so that absorption is possible. This is accomplished by a combination of mechanical and enzymatic processes (Maynard et al. 1979) which can further degrade DNA. These important enzymes (provided by microorganisms) are not secreted by other mammalian tissues (Russell et al. 2008) and may be responsible for the decrease in success rate of species identification in ruminants. Research in the area of ruminant microorganisms and their effect on the degradation of DNA is needed to further explore this relationship.

Table 3: Mean % of sequences identified to species utilizing DNA barcoding in various diet studies

	carnivore[1]	non-ruminant herbivore[2]	Ruminant herbivore[3]
Mean % of sequences identified to species	89%	56.7%	29%

[1]Deagle 2005,2009, Alberdi 2012; [2] Soininen 2009, Valentini 2008; [3]Kowalski 2012, Pegrad 2008, Rave 2010.

This study was consistent with other findings which show that both microhistology and DNA barcoding include a number of disadvantages in their abilities to correctly interpret food habits for herbivores (Giles et al. 2011, Valentini, 2009, Alipayo et al. 1992, Vavra and Holechek 1980). With regard to microhistology, considerable labor and time for actual analysis were involved. Over 750 hours of microscope work and extensive observer training were required in this study in order to develop a plant reference library for epidermal cell characteristics, create an identification key and analyze fecal slides for estimation of known fragments in diet. To identify a plant species by the microhistological technique, more than one characteristic exclusive to each species is necessary for a valid identification (Green et al. 1985). The success of microhistological keys as an aide in the identification of plant cell fragments is attributable to the fact that plant epidermal cells often differ in appearance by a combination of unique characteristics including: cell length and shape, stoma shape, texture, trichome shape and trichome base shape. This study was consistent with previous studies which highlighted that even with proper training in microhistological identification and knowledge of cell characteristics, the absence of a unique combination of epidermal features in some species prohibited identification to the species level (Henley et al, 2001, Smith et al.2002). All species not detected with microhistological analysis in this study fell within the forb and shrub category including *Baileya multiradiata* (present at 0.4% of control diet; detected in only 1 of 4 diets) and *Helianthus annuus* (1% of diet 4

and not detected, falsely detected in diet 1), *Dodonaea viscosa* (1.4% of control diet 4, not detected) and *Isocoma acradenia* (0.8% of control diet 1, not detected). The identification key noted that each of these species contained unremarkable cell characteristics and thinner cell walls which most likely led to the inability of microhistology to detect their presence.

There are a number of features that can potentially contribute towards a lack of unique species identification with DNA barcodes (Ho et al. 2009). Utilizing DNA barcoding, our study was unable to detect an average of 50% of species in control diets including *Sphaeralcea ambigua* (a forb incorporated at 3-8% of control diets), *Atriplex polycarpa*, (a shrub incorporated at 3-4% of control diets) was not detected in three of four diets, *Isocoma acradenia* (shrub) detected in only one of the four diets and *Bouteloua* species (a grass incorporated at roughly 9% of control diets) and not detected by DNA barcoding in diet 1 (Appendix A, table 2). DNA barcoding results indicated uncertainty with regard to detection of *Cynadon dactylon* in diet 1, indicating that these sequences may have come from *Bouteloua* species. *Hordeum jubatum* (1.4% of control diet) was not detected in any of the diets. Ho et al. (2009) utilized DNA barcoding to determine diet overlap of sheep and kangaroos. They reported seven samples which were PCR negative and five samples contained an amplified band that could not be confidently identified. In their study conducted on alpine chamois, Raye et al. (2009) reported that 12%of their DNA sequences had no

correspondence in the trnL P6 Loop database, 49% matched exactly and 39% showed one or two mismatches. Kovach et al. (2003) had limited success (10%) extracting DNA from New England cottontail pellets that were experimentally exposed for a week or longer. According to Morin et al. (2001) and Nsubuga et al. (2004), DNA can produce erroneous results when it is of poor or low quality. Compared with plant material, the conditions for PCR amplification on fecal DNA are technically demanding given the propensity for non-specific amplification (Kovach et al., 2003).

Both microhistology and DNA barcoding were biased in terms of proportion of diet reported. Grasses were consistently overestimated with microhistology and underestimated with DNA barcoding. Control diet mixtures contained an average of 11% grasses and were estimated at 23% for microhistological analysis and 6% for DNA barcoding. Forbs and shrubs, on the other hand, were underestimated with microhistology and overestimated with DNA barcoding. Forbs and shrubs comprised 11% and 8% of control diets (excluding Alfalfa). Utilizing microhistology, forbs and shrubs were estimated at 6 and 5% respectively. With DNA barcoding, both categories were estimated at 26%. As discussed by Mohammed et al. (1995) plant form and the growth stage of the plant determine plant digestibility. Numerous studies utilizing microhistological analysis (Bartolome et al. 1995, Vavra and Holechek 1980, McInnis et al. 1983, Soininen et al. 2009) were consistent with the results of this

study, reporting an overestimation of grasses and underestimation of forbs. This is attributed to the fact that the resistance of grasses to digestion is greater than the resistance of forbs to digestion. By determining and applying appropriate digestion coefficients it is possible to correct for these differences in digestibility (McInnis et al. 1983, Vavra and Holechek 1980). Such correction factors for DNA barcoding, however, have yet to be established.

One of the limitations in this study, with regard to the overestimation of grass by microhistology, is the difficulty encountered in confirming the purity of the hay bales utilized. Invasive grasses may have provided some unwanted variables, thus increasing the percentages and detection of grass within this study. If this were the case, however, a greater report of grasses would have been predicted by DNA barcoding as well. DNA barcoding in this study reported an underestimation of grasses and overestimation of forbs and shrubs (as previously mentioned). This is consistent with Soininen's et al. (2009) results in assessing relative amounts of forage classes. Soininen et al (2009) reported substantial amounts of gramanoids found in the diet of *M. oceconomus* with microscopy which were not evident from DNA analysis. In addition Soininen et al. (2009) reported that the prevalence of non-plant food items (fungi) and various plant structures (bark, root, seed) identified with microhistology could not be identified by DNA barcoding.

Much of the overestimation by DNA barcoding can be attributed to false positive detection within the forb and shrub category. Of particular concern was *Eschscholzia californica* (California Poppy, family Papavaraceae). Although incorporated only in control diet 3 at 1.6%, DNA barcoding results indicated its presence at 9.2% in diets 1 and 2, 13.6% in diet 3 and 14.6% in diet 4 (Appendix B, Table 6). Other forbs detected with DNA barcoding but not present in control diets were *Helianthus annuus,* (Common sunflower, family Asteraceae, diets 1 and 3) *Sisymbrium irio* (London rocket, family Brassicaceae, diets 1 and 2), and *Sonchus oleracea* (common sowthistle, family Asteraceae, diets 2 and 3) all detected at approximately 2% of the diet. According to Raye et al. (2009) a few families (including Asteraceae and Brassicaceae) contain several species which share the same P6 loop sequence leading to either over or under-representation of these species. For DNA barcoding to work successfully, it requires sufficient times since speciation for mutations and/or drift to lead to a set of genetic characters "grouping" conspecific individuals together, separate from other species (Hollingsworth et al. 2009). In clades where speciation has been very recent, or rates of mutations are very slow, barcode sequences may be shared among related species. A particular focus on these species is needed in the future, and additional molecular markers could be implemented to increase the resolution power.

It is becoming increasingly widespread for DNA based identification methods to be applied in studies of wild animal diet (Deagle et al.2009). One of the outstanding issues in recent dietary DNA barcoding studies is the relationship between amounts of various food items consumed and the quantitative data recovered from corresponding dietary samples (i.e., the relative number of sequences generated by high-throughput sequencing of DNA amplified from feces or stomach contents). Discussion of the issues surrounding accuracy of biomass quantification has been included in several recent DNA diet papers (Deagle et al. 2009, Soininen et al. 2009, Valentini et al. 2009). Soininen et al (2009) and Valentini et al. (2009) urge caution in the quantitative interpretation of their DNA barcoding results, stating this attractive perspective still requires empirical validation. However, they also conclude that the approach gives a relatively unbiased picture of food utilization of herbivores. In a study of fur seal diet by pyrosequencing prey DNA in feces (Deagle et al. 2009) the authors outline reasons why a quantitative signature could be inaccurate, but still interpret their tempting data in a semi-quantitative fashion.

CHAPTER 6

CONCLUSIONS

Valentini et al, (2008) indicated that they anticipated that ecologists will increasingly turn to a DNA barcoding approach for diet analysis using fecal material, because in many circumstances it represents the only easy way to identify species. Even though DNA barcoding technology has previously been used to determine components in the diets of several wildlife species (Deagle et al. 2005, Murphy et al., 2003, Soininen et al. 2009), the use of fecal DNA is still only in a developmental stage in terms of determining composition of ruminant diets.

In my study a more accurate mean species composition was reported with microhistological analysis (79%) as compared to DNA barcoding (50%). Microhistological analysis consistently provided a higher species presence by forage class, and for positive species detection microhistology analysis estimated an average of 89% correct detection in control diets, while DNA barcoding estimated 50% correct detection of species.

Some of the major issues leading to poorer response of DNA-barcoding may be: 1) That the exact nature of the rumen bacteria and its effect on degradation of DNA has yet to be determined Russell et al. (2008) and 2) That information on chloroplast content in each feed species (on the copies per cell

basis) was needed to standardize proceedings for quantification in DNA barcoding Ho et al. (2009). For these reasons it is the conclusion of my study that, at its current stage of technological development, DNA barcoding did not enhance the ability to detect plant species in herbivore diets.

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APPENDIX A

FEEDING TRIAL PARAMETERS

Table 4. List of plant species used in the four control diets for the evaluation of the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination. (Names follow those used in USDA PLANTS database http://plants.usda.gov)

Common Name	**Scientific Species**
Grasses	
Needle gramma	*Bouteloua aristoides*
Sideoats gramma	*Bouteloua curtipendula*
Blue gramma	*Bouteloua gracillis*
Bermuda grass	*Cynadon dactylon*
Arizona cottontop	*Digitaria californica*
Foxtail barley	*Hordeum jubatum*
Forbs	
Cuman ragweed	*Ambrosia psilostachya*
Desert marigold	*Baileya multiradiata*
California poppy	*Eschscholzia californica*
Common sunflower	*Helianthus annuus*
Cheeseweed mallow	*Malva parviflora*
London rocket	*Sisymbrium irio*
Common sowthistle	*Sonchus oleracea*
Desert globemallow	*Sphaeralcea ambigua*
Alfalfa	*Medicago sativa*
Shrubs	
Cattle saltbush	*Atriplex polycarpa*
Desert hackberry	*Celtis pallida*
Netleaf hackberry	*Celtis laevigata*
Hopseed	*Dodonaea viscosa*
Eastern Mohave buckwheat	*Eriogonium fasciculatum*
Alkali goldenbush	*Isocoma acradenia*

Table 5. Percent plant species composition of the four control diets used to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

	Plant Composition (%)			
Species	Diet 1	Diet 2	Diet 3	Diet 4
Grasses				
Bouteloua aristoides	0.0	1.5	0.0	2.2
Bouteloua curtipendula	4.0	1.5	3.7	2.2
Bouteloua gracillis	4.0	1.5	4.7	2.2
Cynadon dactylon	2.7	1.5	0.8	4.3
Digitaria californica	1.3	1.5	0.9	0.0
Hordeum jubatum	1.3	0.0	0.3	1.2
Forbs				
Ambrosia psilostachya	1.0	3.3	2.1	1.8
Baileya multiradiata	0.4	0.2	0.5	0.9
Eschscholzia californica	0.0	0.0	1.6	0.0
Helianthus annuus	0.0	1.5	0.0	0.9
Malva parviflora	3.1	3.3	1.6	0.0
Sisymbrium irio	1.8	0.0	0.0	0.0
Sonchus oleracea	0.0	0.0	0.0	1.4
Sphaeralcea ambigua	2.7	8.3	4.7	4.1
Medicago sativa	70.1	70.0	70.0	70.0
Shrubs				
Atriplex polycarpa	2.6	2.2	4.5	2.6
Celtis pallida	1.5	0.6	0.0	0.0
Celtis laevigata	1.1	0.2	0.5	0.5
Dodonaea viscosa	0.0	0.6	0.5	1.4
Eriogonum fasiculatum	1.5	2.4	1.8	2.3
Isocoma acradenia	0.7	0.0	1.8	2.3

APPENDIX B

STATISTICAL ANALYSIS

Table 6. Mean percent plant species composition of the four control diets as determined using two methods to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

	Species Composition by Diet and Method (%)											
	Diet 1			Diet 2			Diet 3			Diet 4		
Species	Control	Micro-Histo	DNA	Control	Micro-Histo	DNA	Control	Micro-Histo	DNA	Control	Micro-Histo	DNA
Grasses												
Bouteloua aristoides	0	0	0	1.5	3.7	6.2	0	0	0	2.2	4.3	2.7
Bouteloua curtipendula	4.1	7.3	0	1.5	4.6	0	3.7	4.7	0	2.2	3	0
Bouteloua gracillis	4.1	12.2	0	1.5	5.8	0	4.7	7.2	1.7	2.2	4	0
Cynadon dactylon	2.7	2	6.4	1.5	5.3	1.5	0.8	4.9	0	4.3	1.8	0
Digitaria californica	1.4	0.7	1.8	1.5	3.2	0	0.9	2.3	1.7	0	4.2	2.7
Hordeum jubatum	1.4	1.1	0	0	0	0	0.3	2.7	0	1.2	6.3	0
Grass Totals	13.7	23.3	8.2	7.5	22.6	7.7	10.4	21.8	3.4	12.1	23.6	5.4
Forbs												
Ambrosia psilostachya	1	1.2	0	3.3	0.8	0	2.1	0.6	1.7	1.8	1	5.3
Baileya multiradiata	0.4	0	1.8	0.2	0.1	1.5	0.5	0	0	0.9	0	0
Eschscholzia californica	0	0	9.2	0	0	9.2	1.6	1	13.6	0	0	14.7
Helianthus annuus	0	0.1	1.8	1.5	2.6	3.1	0	0	1.7	0.9	0	1.3
Malva parviflora	3.2	2.8	12.8	3.3	2.8	7.7	1.6	3.1	0	0	0.3	6.7
Sisymbrium irio	1.8	0.7	3.7	0	0	1.5	0	0	0	0	0	0
Sonchus oleracea	0	0	0	0	0	1.5	0	0	1.7	1.4	0	2.7
Sphaeralcea ambigua	2.7	1.3	0	8.3	2.2	0	4.7	1.7	0	4.1	3.1	0
Medicago sativa	70	63.4	36.7	70	64.9	43.1	70	67.2	57.6	70	68.5	32
Forb Totals (Less Mesa)	9.1	6.1	29.3	16.6	8.5	24.5	10.5	6.4	18.7	9.1	4.4	30.7

Table 1. Continued.

	Species Composition by Diet and Method (%)											
	Diet 1			Diet 2			Diet 3			Diet 4		
Species	Control	Micro-histo	DNA	Control	Micro-histo	DNA	Control	Micro-histo	DNA	Control	Micro-histo	DNA
Shrubs												
Atriplex polycarpa	2.6	4.2	0.0	2.2	1.9	0.0	4.5	4.3	0.0	2.6	1.1	1.3
Celtis palida	1.5	0.7	6.4	0.6	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Celtis laevigata	1.1	0.6	0.0	0.2	0.2	3.1	0.5	0.3	1.7	0.5	0.2	5.3
Dodonaea viscosa	0.0	0.0	0.0	0.6	0.3	0.0	0.5	0.1	1.7	1.4	0.0	0.0
Eriogonum fasiculatum	0.8	0.0	0.0	0.0	0.0	3.1	1.8	0.7	0.0	2.3	0.3	0.0
Isocoma acradenia	1.5	0.1	19.3	2.4	1.7	18.5	1.8	0.2	16.9	2.3	0.3	25.3
Shrub Totals	7.5	5.6	25.7	6.0	5.1	24.7	9.1	5.6	20.3	9.1	1.9	31.9

Table 7. Analysis of variance of species presence of known diets to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Source	Df	Sum of Squares	Mean Squares	F value	Pr (>F)
Block	3	3.17	1.057	2.52	7.48E-02
Method	2	512.67	256.335	611.65	8.33E-27
Diet	3	13.83	4.610	11.00	3.68E-05
Method by Diet	6	27.67	4.612	11.00	1.02E-06
Residuals	33	13.83	0.419		

Table 8. Analysis of variance of forage class presence of known diets to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Source	DF	Sum of Squares	Mean Squares	F Value	Pr>F
Block	3	1.687	0.562333	3.564874	2.44E-02
Method	2	170.792	85.396	541.3621	5.90E-26
Diet	3	3.632	1.210667	7.674938	5.03E-04
Forage Class	2	27.167	13.5835	86.11166	8.01E-14
Method x Diet	6	7.264	1.210667	7.674938	3.28E-05
Method x FC	4	40.667	10.16675	64.45141	3.98E-15
FC x D	6	4.056	0.676	4.285456	2.67E-03
M x D x FC	12	11.111	0.925917	5.869785	2.54E-05
Residuals	105	16.563	0.157743		

Table 9. Analysis of variance of percent composition by forage class in known diets used to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination

Source	df	Sum Sqr	Mean Sqr	F	Pr>F
Block	3	205	68.333333	0.16315	9.20E-01
Method	2	162	81	0.1934	8.25E-01
Diet	3	943	314.33333	0.75051	5.30E-01
Forage Class	2	1.5E+07	7680578	18338.2	5.45E-51
Method x Diet	6	472	78.666667	0.18783	9.78E-01
Method x FC	4	1076140	269035	642.351	8.70E-31
F C x D	6	57640	9606.6667	22.937	1.83E-10
M x D x FC	12	89846	7487.1667	17.8764	4.31E-11
Residuals	105	43977	418.82857		

Table 10. Nonparametric ranked sum analysis of variance for species composition of known diets to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Source	DF	Sum of Squares	Mean Squares	H Statistic	Prob <H
Block	3	73,366	24,455.3	5.8	1.22E-01
Method	2	1,240,988	620,494.0	147.2	1.10E-32
Diet	3	203,114	67,704.7	16.1	1.10E-03
Species	20	35,351,839	1,767,592.0	419.3	2.06E-76
MethodxDiet	6	731,469	121,911.5	28.9	6.31E-05
MethodxSpecies	40	42,269,758	1,056,744.0	250.6	3.82E-25
DietxSpecies	60	229,261,59	382,102.7	90.6	9.79E-01
MxDxS	120	23,454,711	195,455.9	46.4	1.00E+00
Residuals	753	6,859,802	9,110.0		

Table 11. Ranked means of species composition of known diets by method to evaluate the efficacy of DNA and microhistological analysis of ruminant fecal material for diet determination.

Diet	Control	MicroHisto	DNA
Diet 1	54.28	54.29	54.30
Diet 2	53.68	54.29	32.36
Diet 3	54.28	54.29	29.38
Diet 4	54.28	54.29	37.35
Method Mean	54.13	54.29	38.35